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Simultaneous Determination of Lactulose and Mannitol in Urine of Burn Patients by Gas–Liquid Chromatography

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Ratios of lactulose/mannitol excretion in urine have been used to assess the extent of intestinal permeability in various disease and trauma conditions. Reported studies have used this technique to correlate altered gastrointestinal mucosal permeability to translocation of bacteria and endotoxin, leading to occult sepsis in burn patients. Enzymatic methods of analysis for urine concentrations of mannitol and lactulose were used in these studies. We have found that urine from patients with severe burns frequently contains compounds that interfere with the enzymatic methods. We describe using gas–liquid chromatography to determine mannitol and lactulose simultaneously in the urine of burn patients. To avoid the multiple peaks for the anomeric forms of the reducing sugars during precolumn trimethylsilyl derivatization, we converted the sugars to oximes before the silylation step. The method gave good recoveries of mannitol and lactulose added to burn patients' urine samples. Unlike the enzymatic methods, gas–liquid chromatography eliminates the effect of interfering compounds and allows for the simultaneous determination of both sugars in urine samples.

Several investigators have proposed that thermal injury alters gastrointestinal mucosal permeability, causing translocation of bacteria and endotoxin and leading to occult sepsis and multiple organ failure (*1–3*). Ratios of lactulose/mannitol urine excretion have been used to monitor changes in intestinal permeability in various disease conditions (*4, 5*). We have found that the enzymatic methods commonly used to determine mannitol and lactulose in urine [e.g., by Ziegler et al. (*1*)] result in inaccurate estimates of the concentration of these sugars in urine from severely burned patients. Here we describe the application of gas–liquid chromatography to determine simultaneously lactulose and mannitol in the urine of burn patients.

Materials and Methods

Subjects: The seven burn patients used to evaluate the procedure described were from a larger study designed to assess perturbations of intestinal permeability after burn injury. Seven men with burns ranging from 31% to 78% of the total body surface area (mean 58%) were used in this preliminary study to develop our assay technique. On the fourth day after the burns were sustained, the patients were given 5 g of mannitol and 10 g of lactulose in 100 mL of de-ionized water. A 6-h urine collection was initiated after ingestion of the test solution. We added 1 mL of a 200 g/L chlorhexidine solution to the total urine collected. Aliquots of the urine samples were frozen at −20 °C until analyses were performed.

Sample preparation: We added 125 μL of 1 mmol/L methylmannopyranoside reagent as an internal standard to 50 μL of fivefold-diluted (with de-ionized water) urine. The sample and internal standard were dried under nitrogen in a heating block at 75 °C. After these cooled, we added 100 μL of oxime solution (25 mg of hydroxylamine hydrochloride per milliliter of pyridine), capped the samples, and incubated them at 75 °C for 30 min. After letting the samples cool, we added 100 μL of *n*-trimethylsilyl imidazole reagent (Pierce Chemical, Rockford, IL 61105-9976) and incubated the solutions for 15 min at 75 °C.

Standard preparation: Stock solutions of mannitol (5 mmol/L) and lactulose (0.5 mmol/L) were made in de-ionized water. From the stock mannitol solution, we pipetted 20, 40, 60, 80, and 100 μL into disposable injection vials to give standards containing 100, 200, 300, 400, and 500 nmol of mannitol. From the stock lactulose solution, we pipetted 10, 20, 30, 40, and 50 μL into disposable injection vials to give standards containing 10, 20, 30, 40, and 50 nmol of lactulose. We added 125 μL of 1 mmol/L methylmannopyranoside reagent to all standards, then converted the standards to oximes and silylated as described above.

Analysis: We injected 2 μL of prepared sample into a 15 m × 0.53 mm (i.d.) DB-5 capillary column (J & W Scientific, Folsom, CA) installed in a Hewlett-Packard (Palo Alto, CA) gas–liquid chromatograph 5890 Series II (equipped with a flame-ionization detector, an HP7673 autoinjector, and an HP 3396A integrator). The injection temperature was set at 220 °C, the detection temperature at 300 °C, and the flow rate at 9.7–9.9 mL/min. Optimal performance was achieved by setting the initial oven temperature at 150 °C for 6 min, then ramping the temperature to 220 °C at 10 °C/min, followed by an additional ramp to 300 °C at 15 °C/min. The program was terminated at 20 min.

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Human subjects participated in these studies after giving their free and informed voluntary consent. Investigators adhered to AR 70-25 and USAMRDC Reg. 70-25 on Use of Volunteers in Research.

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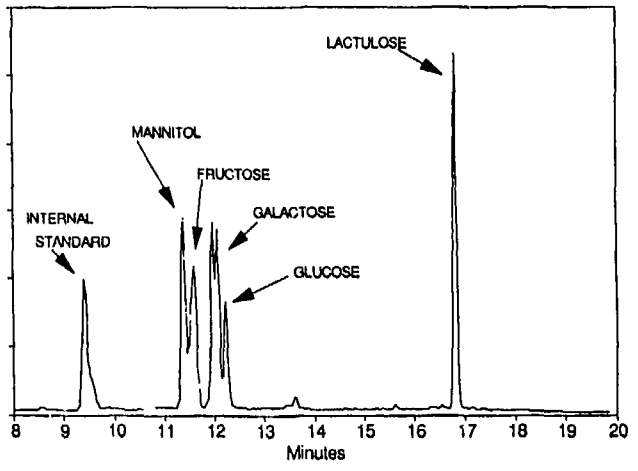

Fig. 1. Chromatogram of a standard containing 100 nmol each of mannitol and lactulose, plus 100 nmol each of added fructose, galactose, and glucose

Results

The chromatogram of a standard solution containing mannitol, fructose, galactose, glucose, and lactulose is shown in Figure 1. None of the three monosaccharides caused interfering peaks with mannitol or lactulose.

Detection of mannitol and lactulose in aqueous standards varied linearly with the quantities injected from 50 to 500 nmol [area = −113 714 + 11 440 (concn), r^2 = 0.99] and from 5 to 50 nmol [area = −20 230 + 10 776 (concn), r^2 = 0.99], respectively. We determined that the minimum detectable concentrations for mannitol and lactulose in urine were 5 and 1 nmol/L, respectively.

To urine samples from three burn patients we added 50, 100, and 150 nmol of mannitol and 5, 10, and 15 nmol of lactulose. The respective mean analytical recovery for mannitol and lactulose for the three samples was 103% and 106%, 97% and 101%, and 101% and 112%.

Urine from one of the burn patients was used to test precision. Six samples were prepared daily for five consecutive days. The mean within-day precision (CV) for lactulose was 5.1%; for mannitol, 2.9%. The between-day CV was 4.5% for lactulose and 0.9% for mannitol.

Chromatograms for urine from a burn patient (45% body surface burn) taken before and 6 h after receiving the mannitol–lactulose dose are shown in Figure 2. The chromatogram of this particular patient shows trace amounts of mannitol, which we often see in burn patients' urine. We confirmed by mass spectroscopy that the peak was mannitol. Subsequent samples analyzed after the mannitol–lactulose dose were corrected accordingly. Many of the injectable treatments received by burn patients contain mannitol as a nonmetabolized additive to maintain physiological osmolarity. Considering that burn patients receive many injections, it is not surprising, given the sensitivity of our method, that trace amounts are detected routinely in patients' urine.

Data from the seven burn patients and seven control subjects are shown in Figure 3. There was no significant difference (Student's t-test) in urine mannitol concentration between the groups (P = 0.44). However, both the lactulose concentration and the lactulose/mannitol ratio were significantly different (P >0.01).

Discussion

Urine samples from patients with severe burns present an analytical challenge to the clinical chemist. Our experience has been that many analytical assays of urine from normal and sick subjects cannot necessarily be applied to urine from burn patients. The enzymatic assay of urinary lactulose is a prime example. The method outlined by Behrens et al. (*6*) involves an enzymatic hydrolysis of lactulose to fructose and galactose, followed by the conversion of fructose to glucose 6-phosphate. Lactulose is measured indirectly by converting glucose 6-phosphate to gluconate 6-phosphate and monitoring at 340 nm the reduction of $NADP^+$.

We have found that urine from patients with severe burns frequently contains compounds that interfere with the enzyme assays used to determine lactulose in urine. Although filtration of the urine through a cation-exchange column removes these compounds, this procedure is time-consuming and increases the chance of dilution errors. Moreover, the enzymatic assay for man-

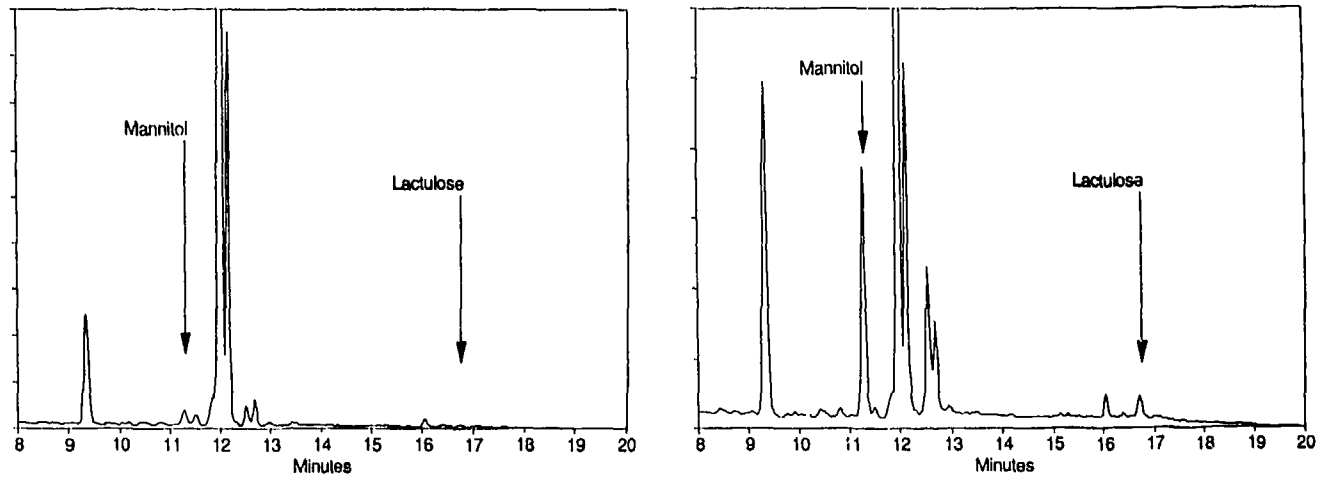

Fig. 2. Chromatogram of urine from a burn patient (45% body surface burn) before (*left*) and after (*right*) receiving the mannitol–lactulose dose

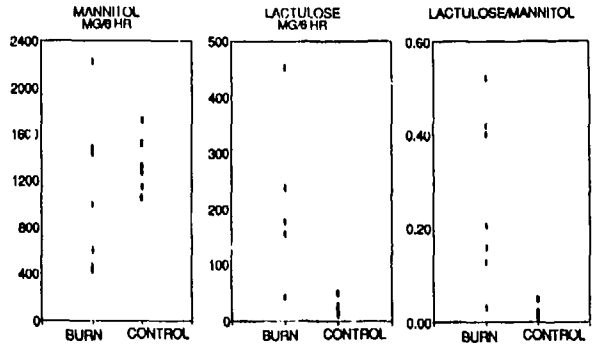

Fig. 3. (*Left*) Amount of mannitol (mg) excreted in the urine of seven burn patients (mean = 1086, SE = 250) and seven control subjects (mean = 1364, SE = 89) 6 h after dosage; (*center*) amount of lactulose (mg) excreted in the urine of seven burn patients (mean = 211, SE = 47) and seven control subjects (mean = 21, SE = 5) 6 h after dosage; (*right*) lactulose/mannitol ratio of seven burn patients (mean = 0.26, SE = 0.07) and seven control subjects (mean = 0.02, SE = 0.01)

nitol requires the synthesis of mannitol dehydrogenase (EC 1.1.1.67), which is not available commercially.

Fleming et al. (*7*) described a method that combines HPLC with pulsed amperometric detection for the simultaneous quantification of lactulose and mannitol in urine. Unlike gas–liquid chromatography, HPLC does not require pre- or post-column derivatization when used with an anion-exchange column. They compared their HPLC method with gas–liquid chromatography, concluding that the former demonstrated better accuracy for lactulose and attributing the problems of gas–liquid chromatography to possible incomplete recovery of the anomers of lactulose.

Laker (*8*) estimated disaccharides in plasma and urine by gas–liquid chromatography, using pre-column trimethylsilyl derivatization of the carbohydrates. A disadvantage of the procedure, however, is that the anomeric forms of the reducing sugars cause multiple peaks in the chromatogram.

Our use of an oxime solution (described in the methods section for gas chromatography, Pierce Chemical Handbook and General Catalog, 1989) eliminates the problem of multiple peaks caused by the anomeric forms. Mannitol and lactulose are first converted to oximes before silylation with trimethysilyl imidazole. Our results demonstrate that the problem with the anomers can be corrected when the initial conversion to oximes is performed before silylation. Furthermore, our gas–liquid chromatography procedure does not require de-salting with ion-exchange resin, a necessary step for the HPLC method.

The mean (SE) lactulose excreted, 21 (5) mg/6 h for the control subjects, is similar to the estimate by Behrens et al. (*6*) of 21 (6) mg/6 h in 13 control subjects. The results from the burn patients support the conclusion by Ziegler et al. (*1*) that burn injury appears to cause increased gastrointestinal mucosal permeability.

We conclude that gas–liquid chromatography is a valid method of simultaneously determining lactulose and mannitol concentrations in urine from normal subjects and burn patients.

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